FORM PTO-1390 REV. 2/01T — U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 08593.0001

U.S. APPLICATION NO. (If known, see 37CFR1.5): 09/914840

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/05594	March 3, 2000	March 3, 1999

TITLE OF INVENTION
APPLICATION OF DNA VECTORS FOR THE TREATMENT OF VIRAL INFECTION

APPLICANT(S) FOR DO/EO/US
Gail LEWANDOWSKI; Gregory A. PRINCE; and John GUZOWSKI

Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☒ is attached hereto (required only if not communicated by the International Bureau.
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C. 154 (d)(4).
20. ☒ Other items or information:
   a. ☒ Copy of cover page of International Publication No. WO00/52185.
   b. ☐ Copy of Notification of Missing Requirements.
   c. ☐

U.S. APPLICATION NO. (If known, see 37CFR 1 5) 09/914840	INTERNATIONAL APPLICATION NO. PCT/US00/05594	ATTORNEY'S DOCKET NUMBER 08593.0001

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTOand all claims satisfied provisions of PCT Article 33 (1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total Claims	87	- 20 =	67	x $18.00	$
Independent Claims	5	-3 =	2	x $80.00	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+$270.00	$
TOTAL OF THE ABOVE CALCULATIONS =					$860.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.					$
SUBTOTAL =					$860.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest priority date (37 CFR 1.492(f)).					$
TOTAL NATIONAL FEE =					860.00
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					$
TOTAL FEES ENCLOSED =					$860.00
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 860.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.
1300 I Street, N.W.
Washington, D.C. 20005-3315

DATED: September 4, 2001

SIGNATURE

Ernest F. Chapman, Reg. No. 25,961

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09/914840

JC03 Rec'd PCT/PTO 04 SEP 2001

PATENT
Attorney Docket No. 08593.0001
CUSTOMER NUMBER 22,852

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. national phase of	)	
	)	
PCT/US00/05594	)	Group Art Unit:
	)	
Inventor: Gail LEWSNDOWSKI et al.	)	Examiner:
	)	
Serial No.: Not Yet Assigned	)	
	)	
Filed: September 4, 2001	)	
	)	
For: APPLICATION OF DNA VECTORS FOR THE TREATMENT OF VIRAL INFECTION	)	

Assistant Commissioner for Patents
Washington, DC 20231

BOX: PCT

Sir:

PRELIMINARY AMENDMENT

Prior to examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

After the title, please add the following new paragraph:

This application is a national phase application of PCT/US00/05594, filed March 3, 2000, which was published in English on September 8, 2000, and claims the benefit of provisional application number 60/123,071, filed March 3, 1999, the content of which is incorporated herein by reference.

REMARKS

No new matter has been introduced by these amendments.

LAW OFFICES
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WASHINGTON, DC 20005
202-408-4000

The examiner is respectfully requested to consider the above preliminary amendment prior to examination of the application.

If there are any other fees due in connection with the filing of this amendment, please charge the fees to Deposit Account No. 06-0916. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our deposit account.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: September 4, 2001

By _____
Ernest F. Chapman
Reg. No. 25,961

EFC/FPD/peg

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Application of DNA Vectors for the Treatment of Viral Infection

GOVERNMENT INTEREST

This invention was made, in part, with Government support under NIH grant no. RO1-MH58374 awarded by the National Institutes of Health. The U.S. Government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to DNA-based constructs and methods for treating viral infections. The preferred constructs are amplicons containing DNA that can be packaged *in situ* by an active viral infection, preferably a Herpesvirus infection, and more preferably, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) infections. The amplicons may express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon-γ (IFN-γ). In a highly preferred embodiment, one or more amplicons express IFN-γ in the epithelial and neural cells of an organism infected with HSV.

The present invention further relates to the use of these constructs for the treatment of viral infections. In a highly preferred embodiment, amplicon DNA is applied topically to a site of active HSV infection and packaged *in situ*. Locally, amplicon-directed cytokine expression reduces the titer of HSV to ameliorate the severity and duration of the active (or productive) infection. Amplicon DNA packaged *in situ* by active HSV is transported to the nervous system. In the nervous system amplicon-directed IFN-γ expresion induces the virus to enter a latent phase. The amplicons of the invention are constructed so that environmental stimuli which reactivate HSV also induce the amplicon-directed expression of IFN-γ. The induced IFN-γ maintains or re-establishes HSV latency, thereby eliminating or reducing recurrent infection.

BACKGROUND

The human Herpesviruses comprise at least eight distinct DNA viruses, including herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and three recently described viruses designated human herpesvirus types 6, 7, and 8. In addition, a large number of Herpesviruses cause disease in non-human hosts including, Equid herpesviruses 1-8; Suid herpesviruses 1 and 2; and Gallid herpes viruses 1-3. Known members of the Herpesviridae family are reviewed in Bernard Roizman, Herpesviridae, Fields Virology, 3d ed., Ch. 71, incorporated herein by reference. All herpes viruses produce both active and latent infections, which permits viral reactivation and recurrent clinical illness throughout the life of the host. The recurrent nature of Herpesvirus infection promotes transmission and contributes to the high prevalence of these viruses.

HSV-1 and HSV-2 (collectively, HSV) have been identified as the causative agents of recurrent oral and genital herpetic lesions. HSV-1 and HSV-2 are highly related and present clinically indistinguishable symptoms. Nevertheless, each exhibits preferential sites of infection such that HSV-1 is primarily responsible for oral-labialis herpes, whereas HSV-2, and to a lesser extent, HSV-1, are responsible for genital and anal herpes.

Clinical manifestations of HSV infection are reviewed in Richard J. Whitley, Herpes Simplex Viruses, Fields Virology, 3d ed., Ch. 73, incorporated herein by reference. HSV possesses both a lytic and a latent phase. The lytic phase begins with exposure to HSV at mucosal surfaces or abraded skin. The virus gains entry into epidermal and dermal cells and initiates viral replication.

The active HSV infections of the lytic cycle frequently result in painful vesicles filled with highly infectious fluid appearing on the dermal and mucosal surfaces of the lips, mouth, pharynx, and nasal passages (oral-labial herpes), and genitals, buttocks, thighs and anal regions (genital herpes). Vesicle formation is frequently preceded by prodrome stage, characterized by a tingling or burning sensation. The infection site reddens (macule or erythema stage) and fluid-filled vesicles appear rapidly after the onset of prodrome, within about six hours in the case HSV-1 infection. Within 3 to 4

days the vesicular fluid becomes pustular due the recruitment of inflammatory cells. On dry, external surfaces, the lesions develop a crusting, scab-like appearance, whereas shallow ulcers are more common on moist mucosal membranes. Untreated, oral infections are generally resolved in 8-10 days, but genital lesions persist for an average of three weeks.

Following primary infection, the virus travels to the neuronal nuclei in the dorsal root ganglia of the sacral, vagal, and/or trigeminal nerves where it remains latent for indefinite periods. While in a latent state, HSV does not produce infectious virus, does not cause disease, and is not transmissible. However, when HSV reactivates, it is transmissible, even if the episode is subclinical or asymptomatic. (Barton et al., Int. J. STD. AIDS 7:229-32 (1996)). Reactivation may be triggered by emotional stress and a variety of environmental factors including, UV light, trauma, heat, fever, colds, fatigue, menses, and certain foods, and usually results in the appearance of new lesions at or near the site of initial infection.

The frequency of reactivation is patient-dependent but may be quite high. About 23% of those infected orally with HSV-1 experience 2 or more recurrences per year. Genital (primarily HSV-2) outbreaks follow a somewhat different pattern. About 500,000 to one million new cases of genital herpes occur in the United States alone. Although only 20-25% of those infected with genital herpes are aware of their condition, more than half of the patients who experience recurrences report at least one episode per month. (Hamuy, R. and B. Berman. 1998. Treatment of Herpes simplex virus infections with topical antiviral agents. Eur. J. Dermatol. 8:310-319).

HSV-1 and HSV-2 are easily transmissible and a large proportion of the population has been infected. Worldwide, 60 to 90% of adults have been infected with HSV-1, and nearly 25% have been infected with HSV-2. HSV is not commonly considered a critical health concern among immunocompetent adults, but for people who suffer frequent oral or genital recurrences, herpes can greatly decrease the quality of life. Although frequently painful, lesions generally heal completely. However, severe or recurrent outbreaks can result in scarring, and infections of the eye may result in corneal blindness. (John Manos, DNA Viruses 1: Herpesviruses, Microbiology and infectious

diseases). Damage to peripheral nerves may result in facial paralysis or Bell's palsy, and movement disorders in children. (Billue, Nurse. Pract. 22:88, 97-100, and 102-105 (1997); Hargrave et al., Dev. Med. Child. Neurol. 40:640-42 (1998)).

In immunocompromised patients, including patients receiving immunosuppressant drugs in association with organ or bone marrow transplants and those with the acquired immunodeficiency syndrome (AIDS), lesions may be chronic, progressive and disabling. Similarly, immunological changes occurring during pregnancy place both the mother and fetus at a high risk of severe infection. Active infection during pregnancy has been associated with spontaneous abortion and occasionally with life-threatening systemic maternal infection. (Fields, Bernard, et al., Fields Virology, Herpes simplex viruses, vol. 2, Ch. 73).

Although the spread of HSV through the nervous system is principally confined to peripheral nerves, HSV is implicated in a particularly lethal form of viral encephalitis. HSV encephalitis is the most common viral infection of the central nervous system, accounting for 10-20% of all isolated viral CNS infections in the United States. Left untreated, HSV encephalitis has a mortality rate of about 70%. Less well recognized CNS associations include mood and behavioral dysfunctions, Alzheimer's disease, and profound global amnesia. (Becker, Y., Virus Genes 10:217-226 (1995); Caparros-Lefebvre, D., et al, J. Neurol. 243:248-256 (1996); Itzhaki, R. F., et al., Lancet 352:238 (1998); Lin et al., Mol. Chem. Neuropathol. 28:135-41 (1996); Kimura, S., et al., Intern. Med. 34:131-133 (1995); Wilson, B. A., et al., J. Clin. Exp. Neuropsychol. 17:668-681 (1995)).

In accord with these CNS disturbances, studies in mice and cotton rats strongly argue that HSV invades the brain during primary ocular HSV infection (Lewandowski et al., J. Neuroimmunol. 55:23-34 (1994) and Lewandowski et al., Proc. Natl. Acad. Sci. USA 90:2005-2009 (1993)), and primary HSV labialis infection (Lewandowski et al. manuscript in preparation). Moreover, because HSV moves along the nerves in both retrograde and anterograde directions, HSV has the potential to spread further into the nervous system with each recurrent episode. Consequently, even if initially limited to the peripheral nervous system, HSV has the potential of invading the brain every time

latency is broken. Notably, no current herpes therapy is designed to keep HSV in a latent state.

Currently, nucleoside analogs have proven somewhat successful at inhibiting HSV replication in the active stages of infection. These compounds include competitive inhibitors of HSV DNA polymerase: the pyrophosphate, Foscarnet, and the acyclic nucleoside phosphonate, cidofovir (De Clercq, E., Intervirol 40:295-303 (1997); Martinez, C. M. et al., Pharmacother. 31:1519-1521 (1997); Oram, R. J., et al, Pediatr. Infect. Dis. J. 17:652-653 (1998)). However, nearly all current herpes therapies rely on acyclic nucleoside analogs such as acyclovir (ACV), valacyclovir, famciclovir, penciclovir (PCV), and ganciclovir (Erlich, K., et al., J. Mad. 166:211-215 (1997); Oram, R. J., et al., Pediatr. Infect. Dis. J. 17:652-653 (1998); Wutzler, P., Intervirol. 40:343-356 (1997)). These compounds are selectively monophosphorylated by the herpesvirus thymidine kinase, but not by the cellular enzyme. The monophosphate nucleotide analog is then further phosphorylated by cellular kinases to the di- and triphosphate forms. The triphosphorylated nucleotide analog interacts with and inhibits the viral DNA polymerase.

In some patients, oral or intravenous administration of nucleoside analog compounds result in a faster healing of lesions, a shortened duration of virus shedding, and a faster resolution of symptoms. In addition, suppressive (daily) oral acyclovir, valacyclovir, or famciclovir may reduce the severity and frequency of recurrent episodes in some patients (Erlich, K. S., West. J. Mad. 166:211-215 (1997); Faro, S., Infect. Dis. Obstet. Gynecol. 6:38-43, (1998)). However, it is likely that suppressive dosage schedules will increase the inherent risk of drug- resistant HSV strains (Reusser, P., J. Hosp. Infect. 33:235-248 (1996)). Indeed, acyclovir-resistant and Foscarnet-resistant HSVs have already been described. (Erlich, K. S., West. J. Mad. 166:211-215 (1997)).

HSV infection can also be treated by topical administration of anti-herpetic drugs. (Hamuy, R. et al., Eur. J. Dermatol. 8:310-319 (1998)). However, topical therapies have at least two challenges. First, the drug must be specially formulated to penetrate the stratum corneum barrier in the skin. Currently, the approved topical formulations include ACV in polyethylene glycol, and ACV or PCV in an aqueous cream. Unfortunately, transdermal delivery in these approved vehicles is very poor and slow. (Freeman, D.

J.,et al., Antimicrob. Agents. Chemother. 29:730-732; Spruance, S. L.,J . Infect. Dis. 146:85-90 (1982)). Secondly, topical application must begin during the brief "prodrome" stage, concurrent with the initiation of HSV replication. Nevertheless, patient-initiated applications frequently do not begin until the lesion becomes visible and the HSV titer is already high.

Consequently, there remains a need in the art for a safe and efficacious treatment for HSV. In particular, there remains a need for treatment which will prevent recurrent outbreaks of active infection, reduce viral transmission, and limit the propagation of infected tissues in a host.

SUMMARY OF THE INVENTION

The present invention addresses these needs by providing a novel system for treating viral infection, preferably, Herpesvirus infections, and more preferably, HSV-1 and HSV-2 infections, using DNA-based constructs. In one embodiment, the constructs are capable of being packaged *in situ* by an active (replicating) viral infection. In another embodiment, the constructs are amplicons programmed to express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon-γ (IFN-γ). In a preferred embodiment, the constructs are both capable of being packaged *in situ* by a replicating virus and expressing anti-viral compounds.

In a highly preferred embodiment, unpackaged amplicon DNA is applied to the site of an active HSV infection and packaged *in situ*. Consequently, amplicon DNA is incorporated into nacent virus particles in lieu of viral genomes. By reducing the concentration of capsids available to the replicating virus, *in situ* packaging of the amplicon attenuates the infection, decreases infectivity and hastens lesion healing. Concurrently, amplicon-directed IFN-γ expression interferes with viral replication, attenuates the on-going infection, and accelerates the onset of latency. In a highly preferred embodiment, amplicon DNA also remains resident in neuronal cells, the same as, or near to those which harbor latent HSV. The same environmental signals which reactivate the latent HSV virus also induce the amplicon-directed expression of IFN-γ. Consequently, viral latency is maintained or quickly re-established.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 HSV-1(pIFN-γ) is schematically depicted in Figure 1. HSV-1 sequences include an a' region packaging site; an HSV-1 origin of DNA replication (ori_s); a transcriptional unit (HSV-1 c Region) containing an HSV-1 immediate early gene promoter (IE 4/5 promoter) and intervening sequences following the promoter. Non-HSV features include a pBR322 backbone containing a prokaryotic origin of replication (Col E1 Ori) and the ampicillin resistance conferring sequence (Amp); the SV40 polyadenylation site; and a mouse interferon gamma coding sequence (mulFN-γ).

Figure 2 Figure 2 compares the total IFN-γ production in the brains of mice infected with the HSV-2(G) strain of HSV-2 (column 1); mice co-infected with HSV-2(G) and HSV(pIFN-γ) (columns 2-3) DNA; and mice co-infected with HSV-2(G) and control vector DNA, HSV(pLAC) (columns 5-6). Constructs HSV-1(pIFN-γ) and HSV-1(pLac) are designated HSV(pIFN-γ) and HSV(pLac), respectively, in Lewandowski et al., J. Neuroimmunol. 55:23-34 (1994).

Figure 3 Color photographs of HSV-1 induced herpes labialis in the cotton rat model.

Figure 4 HSV-1 titers in lips, trigeminal ganglia and brainstem of HSV-1 infected cotton rats.

Figure 5 Color photograph of immunohistochemically stained samples from cotton rats infected with HSV-1. Dark stain indicates HSV-1 protein detection.

Figure 6 Schematic representation of commercially available pBK-CMV phagemid.

Figure 7 Schematic representation of amplicon HSV-2(pCMV).

Figure 8 Schematic representation of amplicon HSV-2(pCMV-LacZ).

Figure 9 Schematic representation of amplicon HSV-2(pCMV-mulFN-γ).

Figure 10 Schematic representation of amplicon HSV-2(pfos-mulFN-γ).

Figure 11 Schematic representation of amplicon HSV-2(pCMV-crIFN-γ).

Figure 12 Color photographs illustrating the reduction in oral lesion formation upon topical application of HSV-2(pCMV) in the cotton rat model.

DETAILED DESCRIPTION OF THE INVENTION

While not intending to be bound to any one theory in the practice of this invention, the expression vectors, treatment systems, and methods described herein are believed to rely on well known properties of viral replication known to be shared among many viral families of large DNA viruses. Although one of skill in the art will recognize that the present invention may be used to treat a host of infectious viral diseases, in the interests of brevity, the Examples 1-13 are confined to Herpesvirus, in particular, HSV-1 and HSV-2. Consequently, this invention may be best understood in light of the current understanding HSV life-cycle. Details of HSV biology may be found in Bernard Roizman and Amy E. Sears, Herpes Simplex Viruses and Their Replication, Fields Virology Ch. 72 (3d. Ed., B.N. Fields et al., Eds., Lippincott-Raven 1996) (incorporated by reference), and in the other referenced cited herein.

The HSV virion consists of four physical elements: 1) an electron-opaque core, containing approximately 150 kbp of double-stranded, predominantly linear DNA; 2) an icosadeltahedral capsid surrounding the core; 3) an amorphous tegument surrounding the capsid; and 4) a glycoprotein-studded outer lipid envelope.

In clinical infections, the virus attaches to cell-surface receptors, usually at a site of dermal or mucosal abrasion. The viral envelope fuses with the plasma membrane of epithelial cells and the de-enveloped capsid enters the cytoplasm. The capsid is transported to the nuclear pores and the DNA is released into the nucleus where it circularizes. Viral transcription, and capsid assembly take place in the nucleus. New copies of viral DNA are produced by rolling-circle replication, cleaved into monomers and packaged into preformed capsids. Tegument proteins and the lipid envelope are added and mature virus is exported through the endoplasmic reticulum. This active infection process is usually accompanied by the localized cell necrosis, inflammation, and vesicle formation characteristic of clinical lesions. Ultimately, however, the virus is cleared from the inoculation site.

In addition, the HSV viruses are highly neurotropic and can spread from the inoculation site to peripheral sensory nerves. Retrograde transport delivers the viral capsids up the nerve to the dorsal root ganglia. Again, the DNA is released through the

nuclear pores into the nucleus and circularizes. However, unlike the situation in epithelial cells, the HSV DNA may remain latent, exhibiting little or no detectable transcription or replication. Nevertheless, under certain conditions, this apparently quiescent episomal construct can be reactivated. The DNA replicates and new infectious particles are transported back down the peripheral sensory nerves by anterograde transport. Upon reaching the synapses, infectious particles attach to epithelial cells at or near the initial site of inoculation and re-establish an active mucocutaneous infection.

Both the clearance of mucocutaneous infection and the reactivation of latent virus have long been associated with the immune status of the host. (See e.g., J. Lindsay Whitton and Michael B.A. Oldstone Immune Response to Viruses, Fields Virology Ch. 10, (Third Ed. B.N. Fields et al., Eds., Lippincott-Raven 1996) (incorporated herein by reference)). This observation has spawned numerous searches for immunological components involved in virulence. Interferons alpha, beta and gamma have been suggested as treatments for HSV infection. (Hamuy et al., Eur. J. Dermatol. 8:310-9 (1998); Smith et al., Virol. 202:76-88 (1994)).

Recently, it has been discovered that interferons, especially IFN-γ, have a potent inhibitory effect on both HSV replication and transcription termination. (Feduchi, E. et al., Virol. 180:822-825 (1991); Komatsu, T., et al., J. Neuroimmunol. 68:1010-108 (1996); Lewandowski, G., et al., J. Neuroimmunol. 81:58-65 (1998)); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998); Schijns, V., et al., J. Neuroimmunol. 28:1-7 (1990); Svennerholm, B., et al., Arch. Virol. 104:153-156 (1989)). Consequently, the production of one or more interferons at the site of infection would hasten the resolution of an active infection. More importantly, the production of one or more interferons at the site of a latent virus would serve to keep the virus in the latent phase, eliminating or reducing the frequency and/or severity of recurrent episodes and limiting the spread of the virus in the host.

The present invention thus encompasses means for delivering antiviral compounds, preferably cytokines, most preferably IFN-γ, to the site of active and/or latent HSV infection. Although any delivery mechanism is acceptable, it is preferred that

the antiviral compound is a protein expressed at or near the desired site of action. Acceptable delivery systems include amplicons, viruses, defective viruses or defective interfering viruses (DI particles), or combinations thereof, which have been engineered to express IFN-γ, and/or other efficacious compounds. (See, e.g., Donald M. Coen and Robert F. Ramig, Viral Genetics, Fields Virology Ch. 5, (Third Ed. B.N. Fields et al., Eds., Lippincott-Raven 1996); Ho, Methods in Cell Biology 43:191-210 (1994); and U.S. Patent No. 5,672,344, each of which is incorporated herein by reference.)) In a preferred embodiment, the delivery system is an amplicon, more preferably, an amplicon based on HSV-1 or HSV-2 elements.

Specific details of constructing and propagating amplicons based on HSV sequences are available in U.S. Patent Nos. 5,501,979; 5,661,033; 5,672,344; 5,599,691; and in Angela P. Dyer and Frank Tufaro, Herpes Simplex Virus Vectors for Gene Therapy of the Nervous System, Protocols for Neural Cell Culture (2nd ed. S. Fedoroff and A. Richardson Eds., Humana Press, 1997); Ho, Meth. Cell Biol. 43:191-210 (1994); Geller, A. I., et al., Proc. Natl. Acad. Sci. USA 90:7603-7607 (1993); and Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)), each of which is incorporated herein by reference. Additional techniques useful for the construction of amplicons and other DNA constructs may be found in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Vol. 1-3, Cold Spring Harbor Laboratory Press, (1989), incorporated by reference.

In the present context, an amplicon is a plasmid which contains bacterial plasmid sequences which allow prokaryotic amplification, as well as sequences which allow expression and/or replication in eukaryotic hosts, and signals compatible with a viral packaging system, usually a virus-infected cell monolayer. Amplicons are thus designed to mimic the packaging signals of an intact viral genome such that amplicon DNA replaces the viral DNA in nascent viral capsids. Thus, in the case of HSV, because an HSV virion can package 152-154 kb of DNA (the HSV genome), 31-32 copies of a 4.8 kb HSV-based amplicon vector would be packaged per virion.

Generally, packaging is accomplished *ex vivo* and requires a defective "helper" virus to generate the packaging machinery and capsids. Unfortunately, an inherent

failing of existing packaging systems is the production of infectious amplicon particles contaminated with helper virus. Consequently, therapies based on standard amplicon/helper virus systems increase the viral load on the host, and may contribute to cytopathic effects. Although techniques such as using conditionally defective viral mutants are designed to reduce cytopathic effects, it is ultimately desirable to avoid exposing the host to any possible additional pathogen.

In a preferred embodiment of the present invention, the amplicons can function without helper viruses and are applied to the site of an active viral infection as unpackaged DNA. Because the amplicon contains signals which mimic those of the actively replicating virus, the exogenous DNA will be packaged into infectious particles *in situ*. Because *in situ* packaging depends on on-going viral replication, the amplicons are only packaged in infected cells. Consequently, the applied DNA is effectively targeted to those specific cells. In addition, because virions containing amplicon vector DNA are indistinguishable from virions containing viral genomes, the packaged amplicon vectors will spread the same as infectious viral particles.

Moreover, with respect to neurotropic viruses such as HSV, the active lesion provides a portal for both the virus and the amplicon to enter the nervous system. Again, because the packaged amplicon is functionally indistinguishable from the packaged virus, it will be transported to the same or similar sites in the peripheral and central nervous system. Consequently, expression of the DNA of the amplicon in neural tissues will occur specifically in HSV-infected regions, thereby increasing the therapeutic effect and greatly decreasing potential side effects.

This embodiment not only avoids the potential dangers to the host, but simplifies production by eliminating the time, effort and expense of helper virus packaging. In addition, this embodiment provides for targeting to the site of active viral infection, and allows targeting to more remote sites of viral residence. Moreover, because this method does not require storage and application of infectious viruses, it provides a more stable and easily formulated medicament.

HSV-based amplicon vectors of the present invention generally contain the following six elements:

1) A backbone vector (for example, pBR322) with a prokaryotic origin of replication, and an antibiotic resistance-conferring gene (usually β-lactamase). This permits amplicon vector replication in bacteria, thereby facilitating amplicon isolation and purification.

2) The HSV-1 or HSV-2 a' sequence.

3) An HSV-1 or HSV-2 replication origin (either ori_s or ori_L).

4) At least one promoter compatible with mammalian cells driving expression of a transgene.

5) A transgene.

6) A polyadenylation site.

The a' and ori sequences are necessary for the replication and packaging of HSV-based amplicons. The a' sequence serves two functions: HSV replicates by a rolling circle mechanism which produces concatamers of the viral genome; a' provides both the site for cleavage into single genome lengths, and the packaging signal for inserting the unit length DNAs into preformed capsids. The HSV genome consists of two covalently linked components designated L (long) and S (short), each of which contains a corresponding origin of viral replication (ori_s and ori_l, respectively).

Because the a' and ori signals may be poorly recognized by non-type viruses, it is preferred that HSV-1-based amplicon vectors contain an a' sequence and ori_s sequence derived from HSV-1, whereas HSV-2-based amplicon vectors contain an a' sequence and ori_s sequence derived from HSV-2. Although in the present examples, HSV ori_s sequences provides an origin of replication, allowing amplicon vector DNA replication in HSV-infected cells, an ori_L sequence may be substituted. Moreover, as recognized by one of ordinary skill, analogous sequences from other viruses may be substituted to affect packaging into actively replicating non-HSV virions and to treat diseases other than HSV-1 and HSV-2.

HSV-based amplicon vectors can be designed with a variety of promoters for constitutive or induced expression of any potential antiviral protein. Preferred promoters fall into two, potentially overlapping, classes: those that will direct expression of the transgene in epithelial cells and those which are active in neuronal cells.

Given the multiple stages of HSV infection, it is preferred that the anti-viral transgene is constitutively expressed in epithelial cells. Consequently, transgene expression from this promoter will coincide with active infection and diminish as the virus is cleared from the epithelial cells. Transgene expression in the lesion will reduce the severity and duration of an infection, promote healing, and induce the virus to enter latency.

In contrast, to limit adverse neurological consequences, it is preferred that expression in neuronal cells is inducible. Ideally, expression in neuronal cells is stimulated by the same signals which stimulate neuronal HSV to break latency and begin replicating. The amplicon will tend to remain in neurons in a 'latent' state similar to HSV, nevertheless, the amplicon-driven transgene will be expressed at the same time that HSV is replicating in the nervous system. Consequently, expression of antiviral compounds in neuronal cells will tend to preserve latency and limit the frequency and/or severity of recurrent herpetic outbreaks.

Thus, the preferred embodiments of the invention comprise DNA-based therapies for HSV infections that will be effective during symptomatic and asymptomatic episodes. Most notably, the inducible neuronal expression of anti-viral proteins will maintain HSV in a virtual latent state. Although expression may be regulated, for example, by using a tetracycline-responsive promoter (Fotaki et al., Gene Therapy 4:901-08 (1997)), it is preferred that the neuronal expression is stress inducible. Any stress-inducible host or viral promoter may be used including, but not limited to, promoters for glucose regulated proteins (grps), inducible heat shock proteins such as *hsc70, hsp*70 and *hsp*90, c-jun, c-fos. In a preferred embodiment, the c-fos gene promoter, which is activated during stress and primary HSV-1 infections, is used to drive inducible neuronal expression of anti-viral proteins. (Gieroba et al., Brain Res. 675:329-32 (1995); Senba et al., Neurosci. Res. 29:183-207 (1997); Sharp et al., Neurotoxicol. 15:51-59 (1994); and Tyrell, EXS. 77:255-71 (1996)).

The amplicons of the invention are programmed to express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon-γ (IFN-γ). Other useful anti-viral compounds which may be expressed alone or in combination with

themselves, and/or with IFN-γ, include IFN-α, IFN-β, TNF-α, TNF-β, TGF-α , and anti-viral antibodies. (Wong et al., Nature 323:819-822 (1986); Lu et al., J. Invest. Dermatol. 108:803-808 (1997); Kumano et al., Antiviral Res. 7:289-301 (1987); and Yamada et al., Arch. Virol. 99:101-109 (1988)).

One highly preferred embodiment comprises a HSV-1 or HSV-2-based amplicon containing: 1) a constitutive CMV immediate early gene promoter driving IFN-γ expression; and 2) a second HSV-based amplicon of the same HSV type, containing the stress-inducible c-fos gene promoter driving IFN-γ expression. Alternatively, both the constitutive and stress- promoters may be constructed on a single HSV-1 or HSV-2-based amplicon.

In this highly preferred embodiment, inoculation at the site of viral replication initiates a four tier anti-viral therapy:

First, IFN-γ expression from the CMV promoter exerts an antiviral effect at the site of active infection. Increasing IFN-γ levels repress the CMV promoter reducing amplicon-directed IFN-γ production in the epithelial cells. However, because IFN-γ protein has a half-life of 48 hours, it continues to exert an antiviral effect for the duration of the outbreak.

Second, the replication machinery of the resident virus packages the amplicon in HSV virions *in situ*. Each virion that packages amplicon DNA becomes replication-deficient and reduces the viral load on the host. Moreover, the infectious titer at the mucosal membrane is decreased, thereby reducing the likelihood of transmission.

Third, HSV virions containing amplicon DNA are transported into the nervous system. The expression of IFN-γ in the nervous system expedites HSV replication termination and establishment of latency.

Fourth, as the host is exposed to emotional or physical stress (sunlight, fever, immunosuppression, etc.), cellular events occur that cause the latent HSV to reactivate and begin to replicate. The same stressors that reactivate latent HSV will activate the c-fos gene promoter in the "latent" HSV-based amplicon vector. The activated c-fos promoter will drive IFN-γ production, which will rapidly terminate the HSV DNA replication cycle. Consequently, no HSV particles will be made, and HSV will quickly

return to a latent state. This will potentially keep HSV in a virtual latent state and block asymptomatic shedding of the virus.

The amplicon DNAs of the present invention may be applied to any stage of an active, primary or recurrent, episode of herpes labialis or genitalis, including vesicular (papule, vesicle, and pustule) and crusting stages. However, it is preferred that the DNA be applied early in the virulence cycle during prodrome (throbbing-tingling) and/or erythyma (macule) stages. Although topical application is preferred, the amplicon may be administered by any method including subcutaneously, intradermally, intravenously, intraperitoneally, by inhalation or lavage, orally, mucosally, intranasally, or by targeted injection into a lesion or affected body site, including dermal and mucosal tissues, eye, brain, ganglia and other neural tissues.

The DNA-containing compositions of the invention may be applied alone, complexed, suspended, or dissolved in a carrier. An efficacious amount of a DNA-containing composition and carrier comprises a preparation for the treatment of a viral disease or condition. Acceptable liquid carriers or carrier components include: water, polyethylene glycol, dimethyl sulfoxide (DMSO), oils, (including petroleum oil, animal oil, vegetable oil, peanut oil, soybean oil, mineral oil, sesame oil), or mixtures thereof, alone or in combination. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, 18th Edition (A. Gennaro, ed., Mack Pub., Easton, Pa., 1990), incorporated by reference. Carriers can be in the form of mists, sprays, powders, waxes, cremes, salves, ointments, patches, poultices, films, or cosmetic preparations. The DNA composition may also include topical anesthetics such as lidocaine, xylocaine and the like, flavorants, emulsifiers, antibiotics, and known or suspected anti-viral compounds including, but not limited to, idoxuridine, interferons, caffeine, anti-viral antibodies, glutaraldehyde, trifluorothymidine (TFT), nonoxynol-9, acyclovir, penciclovir, famciclovir, valaciclovir, cidofovir, brivudin, sorivudine, n-Docosanol, (S)-1-(3-hydroxy-2-phosphonylmethoxypropl) cytosine (HPMPC), arildone, penciclovir, ribonucleotide reductase inhibitors, SP-303 from *Croton iechler*, Foscarnet, tromantadine, or 2-(N,N-dialylaminomethylene)-9[[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl]guanine (U.S.

Patent No. 4,461,757), tumour necrosis factors alpha and/or beta, ascorbic acid, povidone-iodine, and the like.

Because the amplicon DNAs of the invention are preferably administered topically, it is preferred that the DNA is complexed with, dissolved, or suspended in a delivery vehicle that both facilitates DNA transfer through the stratum corneum to the living cells of the lower epidermis, and promotes the uptake of the DNA into the cells. Since 1966, DMSO, an absorption-enhancing agent, has been investigated as a topical delivery system for nucleoside analogs (Becker, Y., Virus Genes 10:217-226 (1995)). In Europe, idoxuridine (IDU) in DMSO (80%) is currently in use for the treatment of herpes labialis, genitalis, and zoster. PEI is a cationic polymer that acts as a DNA "sponge" and efficiently delivers DNA into cells. PEI and similar polycationic polymers can be used to transfect DNA into cells both in vitro and in vivo (Abdallah, B., A., et al., Human Gene Ther. 7:1947-1954 (1990); Boletta, A., et al., Human Gene Ther. 8:1243-1251 (1997); Boussif, O., et al., Proc. Natl. Acad. Sci. USA 92:7297-7301 (1997); Goldman et al., Nature Biotech. 15:462-467 (1997)), and has been used to deliver HSV-based amplicon DNA into HSV-infected ocular cells in mice. (Ho, D. Y., Methods in Cell Biol. 43:191-210 (1994); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1994)). Consequently, preferred carriers comprise DMSO and PEI, alone or in combination.

The invention also relates to the treatment of a patient or organism by administration of an efficacious amount of the DNA compositions described herein. A patient or organism is hereby defined as any person or non-human subject in an experimental model, any person or non-human animal in need of anti-viral therapy, or any subject for whom treatment may be beneficial, including humans and non-human animals. Such non-human animals to be treated include all domesticated and feral vertebrates, preferably but are not limited to mice, rats, fish, including carp, pike, catfish, walleye, turbot and salmon, birds, including ducks, pigeons, chickens, and turkeys, rabbits, hamsters, goats, guinea pigs, deer, donkeys, dogs, cats, swine, horses, cattle, sheep, turtles, lizards, snakes, and non-human primates, including monkeys, macaques, marmosets, chimpanzees, orangutans, gorillas and marmosets.

Treatment comprises effecting any observable or measurable anti-viral effect in a patient or organism. Observable or measurable anti-viral effects include, but are not limited to, reducing viral titer; decreasing viral shedding and infectivity; preventing, limiting, or retarding the spread of the virus in an infected organism; eliminating or reducing the severity and/or duration of an active viral infection, accelerating healing of virus-induced lesions or conditions, promoting, inducing, maintaining or reinforcing viral latency; reducing mortality rates associated with viral infection; and providing prophylactic protection from viral disease, or the effects of a viral disease, by administering the DNA compositions of the invention to an uninfected patient or organism.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention.

Example 1

***In situ* packaging of HSV-based amplicon DNA into HSV virions**

To demonstrate that unpackaged DNA can be packaged *in situ* by an active viral infection, we inoculated DNA of the HSV-1-based amplicon vector HSV-1(pIFN-γ) (Figure 1) into the eyes of HSV-2 infected mice. The construction of HSV-1(pIFN-γ) and the intraocular model of infection are described in detail in Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998) and Lewandowski, G., et al., J. Neuroimmunol. 55:23-34 (1994), respectively. Briefly, the vitrious compartments of 25 BALB.cByJ mice (6-8 weeks of age) were infected with HSV-2(G) (2×10^4 pfu = $2300 \times LD_{50}$). HSV-1(pIFN-γ) DNA was administered intraocularly to ten mice at 6h pre-inoculation and to five mice at 16h postinoculation. The inoculi were formulated to contain 3 μg of an HSV-1-based amplicon plasmid expressing IFN-γ (HSV-1(pIFN-γ) mixed with (I) 5% glucose-saline, or (ii) 9 equivalents of polyethylenimine (PEI), a transfection agent, in the same vehicle.

As expected, the ten HSV-2-infected mice treated with vehicle only died by day 5. By contrast, the five HSV-2-infected mice treated postinoculation with either HSV-1(pIFN-γ) DNA formulation survived 9-10 days. Of the ten HSV-2-infected mice treated with HSV-1(pIFN-γ) DNA pre-inoculation, all survived at least 11 days and 33%

completely recovered. These results indicated that a single administration of HSV-based amplicon plasmid (DNA) expressing IFN-γ could prolong survival of HSV-2-infected mice.

Although the increased survival of HSV-1(pIFN-γ)-treated animals suggests that the amplicon may have been packaged *in situ* by the actively replicating HSV-2 virus, *in vitro* studies (not shown) indicate that HSV-2 virions inefficiently package and replicate amplicon based on HSV-1 DNA. This is most likely due to the mismatch of HSV-2 virions with HSV-1 a' and/or HSV-1 ori_s sequences.

Example 2

HSV-based amplicon DNA is transported into the nervous system where it expresses high levels of IFN-γ

HSV-1(pIFN-γ)-containing virions have previously been demonstrated to be transported from the eye to the brain of HSV-2-infected mice (Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)). To demonstrate neuronal transport and expression, HSV-1(pIFN-γ) and HSV-1(pLac) DNAs (Geller and Breakefield, Science 241:1667-79 (1988), were packaged into HSV-1 virions *in vitro* (Ho, D. Y. , Methods in Cell Biol. 43:191-210 (1994); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)). Mice were then co-inoculated with amplicon DNA and HSV-2 virus by direct injection into the vitreous compartment of the eye. At various times postinfection IFN-γ production was measured in the brain using an ELISA kit (Genzyme Corp., Kendall, MA). An acceptable ELISA assay is also described in Antibodies: A Laboratory Manual, (Harlow & Lane eds., 1988), Cold Spring Harbor Laboratory Press (incorporated by reference).

As shown in Figure 2, very little IFN-γ was detected in the brain during HSV-2-infection (hatched bar). However, high IFN-γ levels were detected in the brains of HSV-2-infected mice co-inoculated with HSV-1(pIFN-γ) (solid bars), strongly suggesting that HSV virions containing HSV-based amplicon vector DNA are transported from the periphery into the nervous system.

Example 3

Experimental models of herpes labialis and herpes genitalis.

Numerous models of HSV pathogenesis are known in the art and are briefly described in Richard J. Whitley, Herpes Simplex Viruses, Fields Virology, 3d. ed., Ch. 73, each of which may be used in the practice and refinement of this invention. Although the ocular model used in the above demonstrations is useful for studying certain neurotropic effects, it provides only a limited representation of the clinical course of HSV infections. Moreover, rapid destruction of non-replicating retinal cells in the HSV-2 ocular model severely limits HSV-based amplicon DNA cellular uptake and forbids multiple applications of HSV-based amplicon vector DNA. Consequently, this application presents novel experimental models of herpes labialis and herpes genitalis in cotton rats and mice which are particularly useful in describing the invention disclosed herein.

Herpes labialis models were established in inbred male and female Sigmodon hispidus cotton rats and BALB.cByJ mice (6-8 weeks of age). Although both mice and cotton rats are susceptible to herpes labialis, infectious virus titers in the mouse nervous system are higher than those in the rat nervous system. In addition, the process of HSV-1 induced lesion formation and healing in mice requires >15 days, as compared to 10 days in the rat. Consequently, the progression of herpes labialis (shedding, lesion formation, and resolution) in cotton rats provides a better model for clinical human disease.

Although the time course of the herpes labialis in cotton rats closely simulates human condition, the wealth of immunological and neurobiological reagents for mice enable a more detailed examination of changes in the immune response and nervous system after application of the instant constructs. Consequently, information obtained by testing the constructs in both systems may be useful for the design of safe and efficacious human treatments. Such treatments may be analyzed in both clinical studies and in human modes of labial and genital herpes such as those described in Rooney et al., Lancet 338:1419-22 (1991) and Rooney et. al., J. Infect Dis. 166:500-506 (1992), both of which are incorporated by reference.

Animals are anesthetized by methoxyflurane inhalation and the center of the lower lip is scratched with a 21-gauge needle. The scratched area is swiped with a sterile cotton swab soaked in HSV-1 inoculum (1×10^7 pfu/ml). The animals are then characterized with respect to: (**1**) lesion formation and healing, (**2**) infectious virus recovery from lips, trigeminal ganglia and brains, and (**3**) immunohistochemical detection of HSV antigens in lips, trigeminal ganglia and brains

Example 4

HSV-1 induced herpes labialis in cotton rats and mice

The progression of HSV-1 induced herpes labialis in the cotton rat model is shown in Figure 3. The prodrome and erthyma stages occurred at 1d-2d (days postinoculation) (Panel A). Lip lesions were first visible at 2d-3d (Panel B), and progressed to the vesicle stage between 4d and 5d (Panel C). Finally, the lesions began crusting at 5d-6d (Panel D), and healing was nearly complete by 9d-10d (Panels D and E).

HSV-1 titers in lips, trigeminal ganglia and brainstem were determined by standard plaque assay and the results plotted in Figure 4. HSV titers in the lips peaked at 2d (Panel A). The sharp decrease in HSV-1 titers at 3d was indicative of the viral replication termination. The low HSV titers at 5d-7d coincided with viral clearance. These data are in good agreement with the clinical stages of herpes labialis. Specifically, HSV replication and shedding were rapid, and occurred during the first 2-3 days, whereas viral clearance and lesion healing required an additional 7 days.

HSV replication in the trigeminal ganglia began at 2d, peaked at 4d, and declined sharply at 6d (Panel B). In the brainstem, HSV replication occurred through 5d, and was decreased by 6d (Panel C). Infectious HSV was no longer detectable in the trigeminal ganglia and brain at 14d. The HSV recovery time courses suggest that HSV travels from the lip into the trigeminal ganglia, and then from the ganglia into the brain.

Immunohistochemical analysis of excised tissues demonstrates the expression of HSV-1 proteins in infected tissue (Figure 5). HSV proteins are abundant in the lip at 1d (Panel A), and are still detectable at 5d during lesion healing (Panel B). As

anticipated, at 5d, we detected HSV in the trigeminal ganglia neurons (Panel C), and tract (Panel D). Unexpectedly, we also detected HSV in the brainstem (Panel E) and cerebellum (Panel F).

By 30d HSV establishes a latent infection in the trigeminal ganglia, and brain. The latent infection can be reactivated in cotton rats by immunosuppression with cyclophosphamide (50 mg/kg) and dexamethasone (8 mg/kg). In the cotton rat model, HSV titers were first detected 8 days post- treatment in lips, trigeminal ganglia, and brainstem. (Data not shown).

Similar results were obtained using BALB.cByJ mice. From these data we conclude that HSV inoculation of cotton rats and mice provide excellent models of herpes labialis primary and recurrent infections.

Example 5

Experimental models of HSV-2 induced herpes genitalis

Sigmodon hispidus cotton rats and BALB.cByJ mice are also susceptible to intravaginal HSV-2 inoculation. Female animals (6-10 weeks) are anesthetized by intraperitoneal injections of acepromazine (2.5 mg/kg) and ketamine (25 mg/kg), and intravaginally inoculated with HSV-2(G) (1×10^7 pfu/ml) (20 µl mice, 75 µl rats). Adult female mice are susceptible to vaginal HSV-2 during diestrus and pregnancy, but not at estrus. However, with progestin treatment, the animals are continually susceptible to HSV-2. (Baker, D. A. and S. A. Plotkin. 1978). Vaginal infection in mice by herpes simplex type II is enhanced with progesterone. (Proc. Soc. Exp. Biol. Med. 158:131-134; Baker, D. A., et al., et al., Proc. Soc. Exp. Biol. Med. 158:131-134; Parr, M. B.,et al.; Lab. Invest. 70:369-380 (1994)). Thus, female cotton rats and mice are injected subcutaneously with 0.01 µg of estradiol benzoate in peanut oil, followed 24 h later by subcutaneous injection of 2.0 mg of progestin (Depo-Provera, Upjohn, Kalamazoo, MI) in PBS. The animals are inoculated 5 days later.

In the absence of progestin treatment, 40%-60% of HSV-2-inoculated animals became infected. Progestin treatment increased the susceptibility to intravaginal HSV-2 to 100%. We observed 100% morbidity and mortality in the HSV-2-infected animals.

HSV was recovered from vaginal washes beginning at 1 dpi, and from dorsal root ganglia and spinal cord beginning at 3 dpi. Although the high mortality rate makes this model difficult, it is an excellent paradigm for efficacy testing of anti-herpes therapies.

Example 6

Construction of HSV-2-based amplicon vectors

HSV-2(pCMV)

The HSV-2(pCMV) amplicon vector was constructed as follows: The TK poly(A), neomycin/kanomycin resistance gene (Neo R), and SV40 origin of replication (SV40 ori) sequences of the pBK-CMV phagemid (Stratagene, LaJolla, CA) (Figure 6), were replaced with the β-lactamase promoter and gene (Amp^R) to generate plasmid pBK-CMV/Amp^R. The HSV-2(G) packaging signal sequence (a' sequence, 550 bp), and origin of replication (ori_s, 667 bp) were amplified from HSV-2(G) genomic DNA (ABI, Gaithersburg, MD) using the polymerase chain reaction (PCR). Each sequence was subcloned into the pCR-Script vector. The HSV-2(G) a' sequence was then cloned into the MluI site of pBK-CMV/Amp^R by blunt-end ligation. Likewise, HSV-2(G) ori_s DNA was cloned into the BspLu11I site, to generate amplicon HSV-2(pCMV) (Figure 7).

HSV-2(pCMV-LacZ)

To remove the bacterial LacZ promoter and a portion of the polylinker, HSV-2(pCMV) will be incubated with endonucleases Sal I and Nhe I. Then the psv-β-galalactosidase vector (Promega), which contains the full-length β-gal gene, will be digested with the Hind III endonuclease. The Nhe I and Sal psv-β-gal vector ends will be modified to have blunt ends, and ligated to Nhe I linkers (Stratagene). The modified psv-gaβ vector will be digested with Nhe I and Sal I and ligated with the NheI/Sal I-digested HSV-2(pCMV) vector to create the reporter construct HSV-2(pCMV-LacZ) (Figure 8).

HSV-2(pfos-LacZ)

HSV-2(pCMV-LacZ) will be digested with endonucleases Nsi I and Nhe I. The mouse c-fos promoter will be amplified from mouse (BALB.c) brain DNA using PCR. The forward and reverse primers (Life Technologies, Gaithersburg, MD) are engineered such that the amplified c-fos promoter sequence will contain a 5' NsiI site and a 3' NheI

site. The amplified c-fos promoter will be ligated with the NsiI/NheI digested HSV-2(pCMV-LacZ) amplicon vector, to generate the stress inducible reporter construct HSV-2(pfos-LacZ).

HSV-2(pCMV-muIFN-γ)

The β-gal gene will be removed from the HSV-2(pCMV-LacZ) vector by blunt end digestion with endonucleases Nhe I and Pvu II. The mouse IFN-γ coding sequence will be amplified from the vector pms10 (ATCC, Manassas, VA), using PCR. However, and as understood by those of skill in the art, human IFN-γ sequences could be amplified to provide human therapeutic amplicons. The forward and reverse primers are engineered such that the amplified IFN-γ gene coding sequence will have a 5' Nhe I site and a 3' Dra I blunt end. The amplified mouse IFN-γ will be ligated with the Nhe I-Pvu II digested vector to generate HSV-2(pCMV-muIFN-γ) (Figure 9).

HSV-2(pfos-muIFNγ)

The CMV promoter in HSV-2(pCMV-muIFN-γ) will be replaced with the mouse c-fos promoter, described above, to generate HSV-2(pfos-muIFNγ) (Figure 10).

HSV-2(pCMV-crIFNγ)

The cotton rat (Sigmodon hispidus) IFN-γ coding sequence (crIFN-γ) will be amplified using PCR from a cotton rat cDNA library. The forward and reverse primers will be engineered such that the amplified crIFN-γ gene will have a 5' Nhe I site and a Dra I blunt 3' end. As described in above, the β-gal gene will be removed from HSV-2(pCMV-LacZ), but replaced with the crIFNγ gene, to generate HSV-2(pCMV-crIFNγ) (Figure 11).

HSV-2(pfos-crIFN-γ)

The CMV promoter will be removed from the HSV-2(pCMV-crIFNγ) vector, and replaced with the mouse c-fos promoter, described above, to generate the HSV-2(pfos-crIFN-γ) vector.

Example 7

Construction of HSV-1-based amplicon vectors

HSV-1-based amplicon vectors are constructed essentially as described for the HSV-2-based amplicon vectors from pBK-CMV/Amp^R except that the HSV-1 a' sequence and ori$_s$ sequences are amplified from HSV-1(F) virion DNA (ABI) and used in lieu of HSV-2 sequences.

Example 8

In vitro testing of amplicon constructs

The HSV-1 and HSV-2-based amplicon constructs may be transfected into Vero cells and tested for the production of expression products using standard methods. For example, IFN-γ production from HSV-2(pCMV-mulFNγ) and HSV-2(pfos-mulFNγ) vector DNA's are measured at 24h using an ELISA kit from Genzyme. Similarly, IFN-γ production from HSV-2(pCMV-crIFNγ) and HSV-2(pfos-crIFNγ) vector DNA's is measured at 24h with an ELISA assay optimized to detect Sigmodon hispidus IFN-γ.

Example 9

HSV-2(pCMV) is packaged into HSV-2 virions in vitro

Vero cells were transfected with HSV-2(pCMV) DNA from 10 clones, and then 24h later infected the cells with HSV-2(G) (0.01 pfu/cell). At 48h postinfection, the culture supernatants were transferred to fresh Vero monolayers. After 24h, total DNA was isolated from the cells and samples were then screened for the β-lactamase gene by PCR. The presence of the β-lactamase gene in all clones indicated that the HSV-2-based amplicon DNA was successfully packaged and replicated in HSV-2(G) virions.

Example 10

Optimizing the administration of HSV-based amplicon vector DNA, and *in situ* packaging into HSV virions.

The HSV-based amplicon constructs described above contain both the DNA packaging/cleavage signal and the HSV ori$_s$. Thus, the HSV virions in herpetic lesions will package topically applied HSV-based amplicon DNA. Each HSV virion can package

152-154 kb of DNA; thus, several copies of an HSV-based amplicon vector could be packaged per virion. Clearly, the level of transgene expression (e.g., β-gal or IFN-γ) will depend on the efficiency of HSV-based amplicon DNA packaging into HSV virions. Moreover, the therapeutic effect of the HSV-based amplicon will depend on the level of transgene expression. Consequently, it is desirable to optimize the conditions for *in situ* packaging in the cotton rat and mouse model systems. This will entail determining: 1) an optimal vehicle for topical DNA delivery; 2) the optimal timing and dosing schedule (e.g., prodrome, erythema, papule, vesicle, pustule, crusting, or scab, single vs multiple applications); and 3) the amount in μg of HSV-based amplicon vector DNA that is most efficiently packaged.

The results obtained using the model systems can then be extrapolated to human patients and used to plan safety and efficacy studies for the topical treatment of herpes labialis and gentalis and related conditions.

Vehicles for DNA delivery

The following experiments provide guidelines for testing the efficacy of DMSO and PEI carriers using the HSV-1-based system. The same basic experiment will be done with the HSV-2(pCMV-LacZ) construct in the HSV-2(G) genitalis models, (without virus) and may be modified to test the efficacy of any other formulation.

Topical administration of HSV-based amplicon DNA in DMSO

To determine if DMSO will enhance the absorption and penetration of topical HSV-based amplicon DNA, HSV-l(pCMV-LacZ) and HSV-2(pCMV-LacZ) DNA in various carriers will be applied to uninfected animals with intact lips. Each application will contain a high concentration (5 μg) of amplicon DNA in order to increase the signal for low degrees of penetration.

Animals will be anesthetized by intraperitoneal injections of ketamine (25 mg/kg) and acepromazine maleate (2.5 mg/kg). 5 μl of the following formulations will then be applied to a small area in the center of the lower lips of cotton rats and mice.

Group 1: DMSO (80%)/saline (20%);

Groups 2 & 6: HSV-1(pCMV-LacZ) DNA (2), and HSV-2(pCMV-LacZ) DNA (6) in saline;

Groups 3-5: HSV-I(pCMV-LacZ) DNA in (3) DMSO (80%)/saline (20%); (4) DMSO (40%)/saline (60%); (5) DMSO (10%)/saline (90%);

Groups 7-9: HSV-2(pCMV-LacZ) DNA in (7) DMSO (80%)/saline (20%); (8) DMSO (40%)/saline (60%), and (9) DMSO (10%)/saline (90%).

10 animals in each group will be analyzed at 0.5, 2, 6, 24, and 48 hours post-treatment. Animals will be sacrificed by CO_2 inhalation, and the lower lips removed. The excised lip tissues will be bisected and fixed in a 0.005% glutaraldehyde/2% paraformaldehyde solution for 1 hour at 4°C. After washing in PBS, the tissues will be placed in a β-gal staining solution (35 mM ferricyanide, 35 mM ferrocyanide, 2 mM $MgCl_2$, 0.02% Tween, 0.02% Nonidet P-40, 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase) overnight at 37°C. After the tissues are washed with PBS, blue staining in the tissue will indicate the β-gal expression from the HSV(pCMV-LacZ) constructs. The lateral and ventral distances between the farthest blue staining and the application site will be measured. These values will be used to determine the diffusion/penetration rates and distances corresponding to each formulation. The ANOVA statistical test will be used to determine whether any significant differences occur between formulations. 10 μm cryostat sections from the tissues will be mounted on charged microscope slides and incubated for 30 min. in the β-gal staining solution. β-gal expression and histopathological changes in the tissues will be monitored by light microscopy. An optimal delivery system will be the formulation that significantly increases the penetration distance from the application site.

Topical administration of HSV-based amplicon DNA complexed with PEI

An increase in cellular uptake will increase the amount of amplicon DNA available to be packaged by HSV virions. The amplicon DNA (5 μg) will be mixed with various PEI equivalents (PEI equivalents are based on the nitrogen content of the polymer) ten minutes before application to allow DNA-PEI complex formation. A small scratch centered on the lower lips of anesthetized animals will be made with a 21-gauge needle. The following DNA-PEI formulations will be applied to the scratched areas.

Groups 1 & 6: HSV-1(pCMV-LacZ) (1), and HSV-2(pCMV-LacZ) (6) in saline;

Groups 2-5: HSV-1(pCMV-LacZ) with: (2) 5 PEI equivalents (equ.), (3) 10 PEI equ., (4) 15 P,EI equ., (5): 20 PEI equ.;

Groups 7-10: HSV-2(pCMV-LacZ) with: (7) 5 PEI equ., (8) 10 PEI equ., (9) 15 PEI equ., (10) 20 PEI equ.

Ten animals will be evaluated per group at 24, 48, and 72 hours post-application. Animals will be sacrificed by CO_2 inhalation, and the lower lips will be removed. β-gal expression will be used as an indication of HSV-based amplicon DNA uptake and expression. Five dissected lips per group will be processed for β-gal expression, and prepared for microscopic analysis as described above. Using a light microscope equipped with a 35-mm camera we will obtain photographs and count the blue-stained cells in each sample. β-gal activity will be quantified in the other 5 dissected lips per group. A 10% homogenate (weight/vol.) will be prepared from the dissected lips in phosphate buffer (pH 7.4). After 2 freeze-thaw cycles, cellular debris will be removed by centrifugation. β-gal activity in the resulting sample supernatants will be measured using a colorimetric assay kit (Promega). Sample proteins will be measured using the BioRad assay and β-gal activities calculated as units per mg protein. Data from cell counts and β-gal activity assays will be analyzed using the ANOVA statistical test. Data from groups 1 and 6 will establish the baseline values for cellular uptake of amplicon DNA without vehicle. Cellular uptake values obtain from groups 2-5, and 7-9 will be compared to the baseline values, and will indicate whether PEI offers any improvement over the use saline alone.

Topical administration of HSV-based amplicon DNA complexed with PEI

Once the optimal (Opt.) concentrations of DMSO and PEI carriers have been determined from the above experiments, uninfected cotton rats and mice with intact lips will be tested to determine whether DMSO can facilitate the penetration and cellular uptake of DNA-PEI complexes, without artificially disrupting the lip surface. 5 ug of DNA will be applied to the lower lip of anesthetized animals with a soft applicator brush. The following formulations will be tested. Group 1: DMSO (Opt.)/PEI (Opt.)/saline;

Groups 2-5: HSV-1(pCMV-LacZ) in (2) DMSO (Opt.)/PEI (Opt.)/saline; (3) DMSO (0.5 Opt.)/PEI (Opt.)/saline; (4) DMSO (0.5 Opt.)/PEI (0.5 Opt.)/saline; (5)

DMSO (Opt.)/PEI (0.5 Opt.)/saline; Groups 6-9: HSV-2(pCMV-LacZ) in (6) DMSO (Opt.)/PEI (Opt.)/saline; (7) DMSO (0.5 Opt.)/PEI (Opt.)/saline; (8) DMSO (0.5 Opt.)/PEI (0.5 Opt.)/saline; (9) DMSO (Opt.)/PEI (0.5 Opt.)/saline; Groups 10-11: HSV-1(pCMV-LacZ) in (10) DMSO (Opt.)/ saline; (11) PEI (Opt.)/saline; Groups 12-13: HSV-2(pCMV-LacZ) in (12) DMSO (Opt.)/ saline; (13) PEI (Opt.)/saline. (0.5 Opt. Refers to one-half of the optimal concentration)

Ten animals will be evaluated per group at 2, 6, 24, 48, and 72 hours post-application. Animals will be sacrificed by CO_2 inhalation, and the lower lips will be removed. Data will be collected on: (a) diffusion/penetration distances, (b) qualitative and quantitative analysis of cellular β-gal expression, and (c) β-gal activity levels in tissue homogenates.

Five animals per group will be used to obtain the diffusion/penetration distances and cellular uptake data, and 5 animals will be used for β-gal activity assays. Once again, the penetration distances will indicate the ability of the vehicle to deliver the HSV-based amplicon DNA through the stratum corneum to the living cells. β-gal expression will indicate the ability of the vehicle to enhance cellular uptake of the HSV-based amplicon DNA. Histological data from group 1 will establish whether the vehicle causes severe skin irritation or histopathology. Data from groups 10-13 will establish the baseline values for DMSO and PEI formulations alone. Data obtained from groups 2-9 will be compared to the baseline values and will indicate whether the combination of DMSO and PEI offers any improvement over the use of either vehicle alone. The ANOVA statistical test will be used to determine if any significant differences occur between the formulations.

The basic experiments described above are for the HSV-1(pCMV-LacZ), and HSV-2(pCMV-LacZ) constructs in the labialis cotton rat and mouse models, without HSV. The results of these experiments will be uncomplicated by the presence of herpetic lesion. However, the presence of a herpetic prodrome or lesion may change the requirements of the vehicle formulation. Also, the topical delivery vehicle formulation may differ somewhat for herpes genitalis. Accordingly, the same basic experiments will be done with the following variations: (1) HSV-1(pCMV-LacZ) construct with HSV-1(F)

in the labialis models, (2) HSV-2(pCMV-LacZ) construct in the HSV-2(G) genitalis models, without virus, and (3) HSV-2(pCMV-LacZ) construct with HSV-2(G) in the genitalis models.

Optimization of dosing schedule of HSV-based DNA amplicon for *in situ* packaging.

Herpes labialis and genitalis lesions occur in 8 stages. Infectious virus is present from the prodromal stage through the pustule (late vesicular) stage. However, current topical treatments of HSV-1 and HSV-2 infections administered after early prodromal or macule (erythema) stages are relatively ineffective (Hamuy, R., et al., Eur. J. Dermatol. 8:310-319 (1998); Spruance, S. L., In E. DeClercq (ed.), Martinus Nijhoff Publishing, p. 69-86 (1998); Spruance, S. et al., J. Infect. Dis. 161:191-197 (1990)). Because the HSV-based amplicon DNA therapy of the present invention preferably incorporates *in situ* HSV-based amplicon DNA packaging by HSV virions, the instant therapy may be effective from the prodromal stage through the vesicular stage.

To determine an optimal dosing schedule, 5 µg of HSV-1(pCMV-LacZ) DNA will be prepared in an optimal delivery vehicle and administered at various times pre- and post-HSV-1(F) inoculation. The HSV-1(pCMV-LacZ) in vehicle will be applied to a small area in the lower lip center of anesthetized animals at the following times.

Groups 1-3: (1) 16h pre-inoculation, (2) 6h pre-inoculation, and (3) 2h pre-inoculation. Pre-inoculation applications will allow cellular uptake of the HSV-1(pCMV-LacZ) DNA before the virus is introduced. This is similar to the HSV-based amplicon DNA packaging protocols used in tissue culture.

Groups 4-5: (4) 2h post-inoculation, and (5) 12h post-inoculation. These groups will represent the prodrome stage of herpes labialis; HSV is present and beginning to replicate, but a lesion is not observed.

Groups 6-10: (6) 24h post-inoculation, (7) 48h post-inoculation, (8) 72h post-inoculation, (9) 96h post-inoculation, and (10) 120h post-inoculation. These groups will represent the progressive stages of herpes labialis.

Group 11: 2h pro-mock inoculation. This group will serve as the negative control for *in situ* packaging by HSV virions.

Groups 1-3, & 11 will be sacrificed 48h after inoculation. Groups 4-10 will be sacrificed 48h after amplicon DNA application. The lips will be removed and tissue homogenates (10% wt./vol.) will be prepared in RPMI 1640 with 2% FBS, as described above. All of the sample supernatants will contain HSV-1 virions with infectious DNA. The supernatants will also contain HSV-1 virions with amplicon DNA, if *in situ* packaging of the HSV-1(pCMV-LacZ) DNA has occurred. We will plate the sample supernatants onto confluent Vero cell monolayers in 96 well plates in triplicate. The plates will be placed at 37°C for 48-72 hours, and then processed for β-gal activities and protein concentrations. Because of the large number of samples to process, data collection will be facilitated by use of a microtiter plate reader set at a wavelength of 420 nm. β-gal activities for each group will be compared and analyzed using the ANOVA statistical test.

The experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. As recognized one of skill in the art, the same basic tests may be done with any number of variations including: (1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models, (2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models, (3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models, and (4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

Optimizing the amount of HSV-based amplicon DNA amount for *in situ* packaging

Ideally, the absolute amount HSV-based amplicon DNA that can be packaged *in situ* depends on the DNA to HSV virion ratio. In accord with the results of the preceding experiments, the following amounts of HSV-1(pCMV-LacZ) DNA prepared in the optimal delivery vehicle will be applied to cotton rats and mice.

Group 1: 0.0 µg;

Groups 2-7: (2) 0.5 µg; (3) 1.0 µg; (4) 5.0 µg; (5) 10 µg; (6) 50 µg; (7) 100 µg;

Groups 8-13: The same as groups 2-7, except that HSV-1(pCMV-LacZ) DNA will be applied at a different time post-HSV inoculation.

Ten animals per group will be sacrificed at a predetermined time point post HSV-1(pCMV-LacZ) DNA application. The lips will be removed and tissue homogenates prepared. The supernatants will be analyzed for the presence of HSV virions containing HSV1(pCMV-LacZ) DNA on Vero indicator cells. The β-gal activity values will be plotted

against the HSV-based amplicon DNA doses, and the data will be analyzed for statistical significance using the ANOVA statistical test.

The basic experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. The same basic experiment will be done with the following variations: (1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models, (2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models, (3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models, and (4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

Example 11

Establishing the parameters affecting amplicon packaging, transport, and expression

The greatest therapeutic value of topical HSV-based amplicon DNA for the treatment of herpes involves (1) packaging of HSV-based amplicon DNA into HSV virions at the lesion site, (2) the spread of HSV-based amplicon DNA into the nervous system and 3) transgene expression in neural cells. While IFN-γ may have antiviral actions at the lesion site, the greatest therapeutic potential of IFN-γ is to facilitate the transition from the productive infection to the latent HSV infection in neurons. Accordingly, this experimental series demonstrates that topically applied HSV-base amplicon DNA is packaged *in situ* into HSV virions, then transported along peripheral nerves, and expressed in the nervous system.

The lips of cotton rats and mice will be inoculated with HSV-1(F). HSV-1(pCMV-LacZ) amplicon DNA will then be applied to the lips of Group 1 animals. Group 2 animals will comprise the control. DNA application will use pre-established conditions for optimal *in situ* packaging of HSV-1(pCMV-LacZ) DNA into HSV-1 virions.

The time course of HSV-1(F) spread from the lip to the nervous system has previously been established using immunohistochemical analysis and infectious virus recovery (titration) assays (data not shown). 15 animals from each group will be sacrificed at 4, 5, 6, 7, 14, and 30 days postinfection. At each time point, 5 animals from group 1 and 3 animals from group 2 (controls) will be perfused with 4% para-formaldehyde in phosphate buffer. The trigeminal ganglia and brains will be removed

and placed in an 18% sucrose/phosphate buffer solution overnight at 4°C. Frozen sections of 5 µm will be made using a cryostat, and melted onto charged microscope slides. The sections will be incubated with monoclonal antibodies (mAb) specific for β-gal (Boehringer Mannheim), and the HSV-1 immediate early gene, ICP4 (ABI). Primary antibody binding will be visualized with secondary antibodies conjugated to either Cy3 (red fluorescence) or Cy2 (green fluorescence). ICP4 positive- (red), β-gal positive- (green) and double positive cells will be visualized using fluorescence and light microscopy. Cells infected by HSV-1 virions with HSV-based amplicon DNA will be positive for only β-gal (green staining). Cells infected by HSV-1 virions with HSV-1 DNA will be positive for only ICP4 (red staining). Finally, cells infected with both types of HSV-1 virions will be positive for both β-gal and ICP4 (red-, green-, and orange staining). With these data we will determine if HSV-1 virions containing HSV-based amplicon DNA and HSV-1 virions containing HSV-1(F) DNA are transported to the same or different cells in the nervous system.

In addition, 10 animals in group 1 and 8 animals in group 2 will be sacrificed at each time point by CO_2 inhalation to remove the trigeminal ganglia and the brainstem-cerebellum region of the brains. β-gal activity levels will be measured in the tissue homogenate supernatants from 5 animals in group 1 and 3 animals in group 2 per time point as described above. Infectious virus titers at each time point will be measured in the tissue homogenate supernatants from the other 5 animals in each group. The titers of infectious HSV-1 will be plotted against time postinfection. With these data we will establish: 1) the time courses for HSV-1 infection and β-gal transgene expression in the nervous system; 2) whether transgene expression is simultaneously turned off with the termination of HSV- 1(F) DNA replication, and; 3) if the time course of HSV infection in the nervous system is altered by the presence of HSV-1 virions with amplicon DNA.

The experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. The same basic experiment will be done with the following variations:

1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models;

2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models;

3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models; and

4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

In the herpes genitalis models, the above-described analysis will be done with the lumbosacral dorsal root ganglia, spinal cords, and brainstems. Similar studies will demonstrate transgene (β-gal and IFN-γ) expression during HSV reactivation.

Example 12

HSV-2(pCMV) DNA is packaged *in situ* and transported to neural sites

Eight cotton rats were inoculated with HSV-2 on their bottom lip. Six hours later, rats were anesthetized and the inoculation site saturated with the delivery vehicle solution (80% DMSO and 5% glucose in saline). Immediately thereafter, 10 μl of HSV-2(pCMV) amplicon DNA (5μg) mixed with PEI (9 equivalents) was carefully placed on the inoculation site, using a pipettor. Delivery vehicle and HSV-2(pCMV) vector DNA was topically applied to the inoculation site in this manner twice a day for 5 days.

Four rats were sacrificed on day 5, and 4 rats were sacrificed on day 6. A careful examination of the inoculation sites indicated that 6 of the animals showed no signs of lip lesions at the time of sacrifice. The HSV-2(pCMV) vector does not express IFN-γ or any transgene. Thus, the low incidence of lesion formation in this experiment results because HSV-2(pCMV) DNA competed with infectious HSV-2 DNA for packaging. This effectively reduces the titer of infectious HSV-2 and reduces the likelihood of overt lesions.

DNA was isolated from the trigeminal ganglia (peripheral nervous system) and specific brain regions (brainstem & cerebellum) of all animals and tested for the presence of the β-lactamase (ampicillin resistance) gene by PCR. Three of the animals had detectable β-lactamase DNA in brain and/or trigeminal ganglia. Detection of the β-lactamase gene in the brain and trigeminal ganglia of some HSV-2(pCMV)-treated animals indicates that the amplicon DNA can be packaged *in situ* and transported into the nervous system.

Example 13

Topical application of HSV-2(pCMV) DNA inhibits lesion formation

Seven cotton rats were inoculated with HSV-2 on their bottom lip. Sixteen hours later, rats were anesthetized and the inoculation site saturated with the delivery vehicle solution (80% DMSO and 5% glucose in saline). Immediately thereafter, 10 μl of HSV-2(pCMV) amplicon DNA (5μg) mixed with PEI (9 equivalents) was carefully placed on the inoculation site of four of the rats. Delivery vehicle and HSV-2(pCMV) vector DNA was topically applied to the inoculation site in this manner twice a day for 5 days.

Lip lesion formation for each rat was photographed daily for 5 days. As expected, by day five, the vehicle-treated rats had large herpetic lesions. In contrast, the amplicon-treated rats did not exhibit any obvious lesions.

Figure 12 illustrates this dramatic reduction in lesion formation. The top panels show two of the HSV-2-inoculated rats treated with vehicle only. The bottom panels show two of the HSV-2 inoculated rats treated topically with HSV-2(pCMV) DNA.

All animals were sacrificed on day 5 and DNA was isolated from neural tissues as above. The β-lactamase gene was not detected in the DNA's from HSV-2(pCMV)-treated animals in this experiment.

This specification is most thoroughly understood in light of the teachings of the references cited herein, all of which are hereby incorporated by reference in their entirety.

The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan recognizes that many other embodiments are encompassed by the claimed invention and that it is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

We claim:

1. **An *in situ* method for packaging DNA into a viral particle comprising applying at least one unpackaged DNA construct to the site of an active viral infection in an organism, wherein at least one applied DNA construct is packaged into viral particles.**

2. The method of claim 1, wherein the viral infection is a Herpesvirus infection.

3. The method of claim 2, wherein at least one DNA construct is an amplicon.

4. The method of claim 3, wherein the amplicon comprises sequences compatible with packaging into an HSV virion.

5. The method of claim 4, wherein the amplicon comprises a' sequences compatible with packaging into HSV-1.

6. The method of claim 5, wherein the amplicon further comprises an HSV ori sequence.

7. The method of claim 6, wherein the HSV ori sequence is an HSV-1 ori_s sequence.

8. The method of claim 4, wherein the amplicon comprises a' sequences compatible with packaging into HSV-2.

9. The method of claim 8, wherein the amplicon further comprises an HSV ori sequence.

10. The method of claim 9, wherein the HSV ori sequence is a HSV-2 ori_s sequence.

11. **A method of treating a viral infection comprising the steps of:**

1) applying a composition comprising at least one unpackaged DNA construct to a site of viral infection in an organism, and

2) packaging at least one applied DNA construct *in situ*.

12. The method of claim 11, wherein the viral infection is a Herpesvirus infection.

13. The method of claim 12, wherein the Herpesvirus is HSV-1 and at least one unpackaged DNA construct comprises a' sequences compatible with packaging by HSV-1.

14. The method of claim 13, wherein at least one unpackaged DNA further comprises an HSV ori sequence.

15. The method of claim 12, wherein the Herpesvirus is HSV-2 and at least one unpackaged DNA construct comprises a' sequences compatible with packaging by HSV-2.

16. The method of claim 15, wherein the at least one unpackaged DNA further comprises an HSV ori sequence.

17. The method of claim 12, wherein the infection is an HSV infection and the composition is applied during the prodrome stage.

18. The method of claim 12, wherein the infection is an HSV infection and the composition is applied during a vesicular stage.

19. The method of claim 11, wherein at least one applied DNA construct is capable of replicating in a host organism in the presence of an active viral infection.

20. The method of claim 11, wherein the composition is applied topically.

21. The method of claim 11, wherein the organism is human.

22. **A method of treating a viral infection comprising the steps of:**

1) applying a composition comprising one or more unpackaged amplicons to a site of viral infection in an organism,

wherein one or more of the amplicons encode at least one anti-viral compound, and

2) expressing at least one of the encoded anti-viral compound in the organism.

23. The method of claim 22, wherein the viral infection is a Herpesvirus infection.

24. The method of claim 23, wherein the Herpesvirus is HSV-1.

25. The method of claim 24, wherein one or more amplicons comprise a' sequences compatible with packaging by HSV-1.

26. The method of claim 25, wherein one or more amplicons further comprise a HSV ori sequence.

27. The method of claim 26, wherein the HSV ori sequence is a HSV-1 ori_s sequence.

28. The method of claim 23 wherein the Herpesvirus is HSV-2.

29. The method of claim 28, wherein one or more amplicons comprise a' sequences compatible with packaging by HSV-2.

30. The method of claim 29, wherein one or more amplicons further comprises a HSV ori sequence.

31. The method of claim 30, wherein the HSV ori sequence is a HSV-1 ori_s sequence.

32. The method of claim 22, wherein one or more amplicons are packaged *in situ*.

33. The method of claim 22, wherein at least one anti-viral compound is a cytokine.

34. The method of claim 33, wherein the cytokine is an interferon.

35. The method of claim 34, wherein the cytokine is interferon gamma (IFN-γ).

36. The method of claim 35, wherein the interferon gamma is human interferon gamma.

37. The method of claim 22, wherein at least one antiviral compound is expressed during active infection.

38. The method of claim 22, wherein expression of at least one antiviral compound is constitutive.

39. The method of claim 22, wherein expression of the antiviral compound is stress-inducible.

40. The method of claim 22, wherein at least one antiviral compound is expressed in epithelial cells.

41. The method of claim 22, wherein at least one antiviral compound is expressed in neuronal cells.

42. The method of claim 22, wherein expression of at least one anti-viral compound is directed by a fos promoter.

43. The method of claim 22, wherein expression of at least one anti-viral compound is directed is directed by a CMV immediate early gene promotor.

44. The method of claim 22, wherein the organism is human.

45. The method of claim 22, wherein the organism is a non-human animal.

46. The method of claim 22, wherein the composition is applied topically.

47. The method claim 23, wherein the infection is an HSV infection and the composition is applied during the prodrome stage.

48. The method claim 23, wherein the infection is an HSV infection and the composition is applied during a vesicular stage.

49. **A composition comprising one or more amplicons capable of directing the inducible expression of at least one anti-viral compound in neuronal cells, wherein the expression of the at least one anti-viral compound in neuronal cells decreases the frequency or severity of HSV reactivation in an organism.**

50. The composition of claim 49, wherein the promoter is stress-inducible.

51. The composition of claim 49, wherein the promoter is a fos promoter.

52. The composition of claim 49, wherein the anti-viral compound is a cytokine.

53. The composition of claim 52, wherein the cytokine is an interferon.

54. The composition of claim 53, wherein the interferon is IFN-γ.

55. The composition of claim 54, wherein the interferon if human IFN-γ.

56. The composition of claim 49, wherein one or more amplicons comprises an HSV a' sequence and an HSV ori sequence.

57. The composition of claim 56, wherein the at least one amplicon is capable of being packaged *in situ* by an active HSV-1 infection.

58. The composition of claim 57, wherein the at least one amplicon is capable of being packaged *in situ* by an active HSV-2 infection.

59. The composition of claim 57, further comprising a carrier.

60. The composition of claim 49, wherein the carrier comprises at least one member of the group consisting of DMSO and PEI.

61. The composition of claim 49, wherein the organism is a human.

62. **A method of reducing the frequency or severity of HSV recurrences in an organism comprising administering the composition of claim 49.**

63. The method of claim 62, wherein the composition is applied topically to the site of an active HSV infection.

64. The method of claim 63, wherein the composition is applied during the prodrome stage.

65. The method of claim 63, wherein the composition is applied during a vesicular stage.

66. **A composition for treating HSV infections comprising one or more amplicons programmed to express:**

1) at least one anti-viral compound in HSV infected epithelial cells, and

2) at least one anti-viral compound in HSV infected neuronal cells, wherein amplicon-directed expression of at least one anti-viral compound in HSV infected neuronal cells is inducible.

67. The composition of claim 66, wherein at least one anti-viral compound is a cytokine.

68. The composition of claim 67, wherein the cytokine is an interferon.

69. The composition of claim 68, wherein the interferon is IFN-γ.

70. The composition of claim 69, wherein the interferon if human IFN-γ.

71. The composition of claim 66, wherein neuronal expression is stress inducible.

72. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by a fos promoter.

73. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by a constitutively active promoter.

74. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by the CMV immediate early gene promotor.

75. The composition of claim 66, wherein one or more amplicons comprise unpackaged DNA.

76. The composition of claim 66, wherein one or more amplicons comprises sequences compatible with packaging into HSV-1.

77. The composition of claim 76, wherein the one or more amplicons further comprise an HSV ori sequence.

78. The composition of claim 66, wherein one or more amplicons comprises sequences compatible with packaging into HSV-2.

79. The composition of claim 78, wherein the one or more amplicons further comprise an HSV ori sequence.

80. The composition of claim 66, further comprising a carrier.

81. The composition of claim 80, wherein the carrier comprises at least one member of the group consisting of DMSO and PEI.

82. **A method for treating HSV infection comprising administering the composition of claim 66 to an organism.**

83. The method of claim 82, wherein the organism is human.

84. The method of claim 82, wherein the organism is a non-human animal.

85. The method of claim 82, wherein the composition is applied topically.

86. The method claim 82, wherein the composition is applied during the prodrome stage.

87. The method claim 82, wherein the composition is applied during a vesicular stage.

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **APPLICATION OF DNA VECTORS FOR THE TREATMENT OF VIRAL INFECTION** the specification of which ☐ is attached and/or ☒ was filed on September 4, 2001, as United States Application Serial No. or filed on March 3, 2000 as PCT International Application No. PCT/US00/05594, and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
			☐ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing
60/123,071	March 3, 1999

☐ I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., CUSTOMER NUMBER 22,852**, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewris, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; James B. Monroe, Reg. No. 33,971; Doris Johnson Hines, Reg. No. 34,629; Allen R. Jensen, Reg. No. 28,224; Lori Ann Johnson, Reg. No. 34,498; and David A. Manspeizer, Reg. No. 37,540 and . Please address all correspondence to **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, 1300 I Street, N.W., Washington, D.C. 20005, Telephone No. (202) 408-4000.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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